

**REMARKS**

Claims 54-63 and 66-93 are pending in this application. Claims 1-53 and 64-65 have been cancelled without prejudice or disclaimer to the subject matter recited therein. Claims 54, 61 and 92 have been amended. Claims 67-91 and 93 have been withdrawn as being directed to non-elected subject matter.

Claims 1-53 and 64-65 have been canceled without prejudice or disclaimer, and claims 54, 61 and 92 have been amended, for the sole reason of advancing prosecution. Applicants, by canceling or amending any claims herein, make no admission as to the validity of any rejection made by the Examiner against any of these claims. Applicants reserve the right to reassert any of the claims canceled herein or the original claim scope of any claim amended herein, in a continuing application.

Claim 54 has been amended to recite “[a] method for preserving a biological material selected from the group consisting of cells, cell aggregates, tissue, organs, biological fluids, natural liposomes and synthetic liposomes, the method comprising: (a) adding a preservation solution to said biological material, the preservation solution comprising one or more polyphenols; (b) freeze drying the biological material; and (c) storing the biological material under appropriate storing conditions.” Support for the amendment to claim 54 can be found throughout the specification and claims as originally filed.

Claim 61 has been amended to correct dependency. As amended, claim 61 now depends from claim 60. Support for the amendment to claim 61 can be found throughout the specification and claims as originally filed.

Claim 92 has been amended to recite A method for preserving a biological material, comprising: (a) adding a preservation solution to the biological material, the preservation solution comprising one or more polyphenols and being essentially free from any polyalcohol; (b) freeze drying the biological material; and (c) storing the biological material under appropriate storing conditions. Support for the amendment to claim 92 can be found throughout the specification and claims as originally filed.

The specification has been amended to properly reference the two provisional patent applications from which the present subject matter claims priority. Specifically, the specification has been amended to reference US Provisional Patent Application No. 60/577,210, filed June 7, 2004 and US Provisional Patent Application No. 60/540,557, filed February 4, 2004.

The abstract of the disclosure has been amended to correct a minor typographical error. Specifically, the repeated term "MNC" has been replaced with "HSC." Support for the amendment to the abstract can be found throughout the specification and claims as originally filed.

***I. At page 4 of the Official Action, the Abstract has been objected to.***

Applicants respectfully thank the Examiner for suggesting that the repeated term "MNC" be replaced with "HSC." As suggested, Applicants have amended the abstract accordingly. Accordingly, the Examiner is respectfully requested to withdraw this objection.

***II. At page 4 of the Official Action, the specification has been objected to.***

Applicants respectfully thank the Examiner for the suggestion to amend the priority information into the present specification. As requested, the specification has been amended to reference US Provisional Patent Application No. 60/577,210, filed June 7, 2004 and US Provisional Patent Application No. 60/540,557, filed February 4, 2004. Accordingly, the Examiner is respectfully requested to withdraw this objection.

***III. At page 4 of the Official Action, claims 61 and 92 have been rejected under 35 USC § 112, second paragraph.***

The Examiner asserts that claim 61 is indefinite because there is no antecedent basis for the term “the macromolecule.” The Examiner asserts that claim 92 is indefinite because the meaning of the phrase “essentially free.”

In view of the remarks set forth herein, this rejection is respectfully traversed.

Applicants respectfully submit that claim 61 has been amended to depend from claim 60. Applicants note that claim 60 provides sufficient antecedent basis for the phrase “the macromolecule,” as recited in claim 61. Accordingly, Applicants submit that claim 61 is clear and definite within the meaning of 35 USC § 112.

With regard to claim 92, the Examiner asserts that the term “essentially free” is indefinite because the specification provides two conflicting definitions of this term. Applicants respectfully disagree with the Examiner because a person

of ordinary skill in the art reading the specification would understand the two meanings of “essentially free” provided in the specification as similar and not conflicting at all.

Applicants submit that the first definition of “essentially free” in the specification is in reference to the amount of polyalcohol used in preservation of the biological material. As evidence of this Applicants note that paragraph [0056] of the present published application provides that:

In some embodiments of the invention, small amounts of one or more polyalcohols, e.g. glycerol, that are conventionally used in preservation of biological material, particularly in preservation procedures involving cooling to below freezing, may be included in the preservation solution. However, it was surprisingly found in accordance with the invention that it is possible to preserve biological material even without use of polyalcohols in the preservation solution. The preservation solution used in accordance with the invention is thus preferably essentially free of any such polyalcohols, e.g. glycerol. The term ***“essentially free” should be understood as meaning free of any polyalcohols such as glycerol, or that any amount of polyalcohol present in the solution is so low so as not to have any effect on the preservation process, on the outcome of the preservation process or on the properties of the biological material (for example the viability of living matter, e.g. cells, if such are included in such material) after it is taken out of the preservation conditions.*** (Emphasis Added).

Additionally, the second occurrence of a definition of the term “essentially free” is in reference to the amount of additive used. As evidence of this, Applicants note that paragraph [0075] of the published present application provides that:

According to yet another aspect, the present invention also provides biological material comprising viable biological material and one or more polyphenols. Such polyphenols may include one or more catechins, such as EGCG, or be derived from green tea

extract (GTE). One of the benefits of the present invention is the biological material may be essentially free of additives that are known to be hazardous or toxic, such as DMSO or polyalcohols such as glycerol and other undesired chemicals such as ethylene glycol, propylene glycol and other alcohols, butandiol and methanol). The term **"essentially free" means that the additive are less than 5%, preferably less than 3%.** The biological material of the present invention may comprise cells selected from RBC, WBC, MNC, UCB, HSC and bacteria, and may have any temperature, above or below zero including temperatures of cryopreservation or room temperature, providing that the biological material is viable. In addition, the biological material may be in a dry state or almost fully dry (comprising 10% or less of its original water content). (Emphasis Added).

Applicants submit that reading the claims in view of the specification a skilled artisan would understand the meaning of the term "essentially free" as meaning that, for example, polyalcohols are not present or present in a very small amount so as to prevent any undesired effects. Accordingly, Applicants submit that claim 92 is clear and definite within the meaning of 35 USC § 112.

It is submitted that claims 61 and 92 are clear and definite within the meaning of 35 USC § 112, second paragraph. Therefore, the Examiner is respectfully requested to withdraw this rejection.

**IV. At page 4 of the Official Action, claims 54-64 have been objected to as being informal.**

The Examiner Asserts that claim 54 is improper because the phrase "comprising one or more polyphenols" should be moved so it occurs immediately after "preservation solution." Applicants respectfully note that this objection has been obviated by the amendment to claim 54. Accordingly, the Examiner is respectfully requested to withdraw this objection.

**V. At page 5 of the Official Action, claim 64 has been objected to.**

The Examiner asserts that claim 64 is a substantial duplicate of claim 63. Applicants submit that in view of the cancelation of claim 64, this objection has been rendered moot.

**VI. At page 6 of the Official Action, claims 54-59, 63 and 64 have been rejected under 35 USC § 102(b) as being anticipated by Gen (US Patent Publication No. 2002/0119946).**

The Examiner asserts that Gen teaches each and every element of claims 54-59, 63 and 64.

In view of the remarks set forth herein, the rejection of claims 54-59 and 63 is respectfully traversed. Additionally, Applicants respectfully submit that the rejection of claim 64 has been rendered moot by the cancellation thereof.

The test for anticipation is whether each and every element as set forth is found, either expressly or inherently described, in a single prior art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987); MPEP § 2131. The identical invention must be shown in as complete detail as is contained in the claim. *Richardson v. Suzuki Motor Co.*, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989); MPEP §2131. The elements must also be arranged as required by the claim. *In re Bond*, 15 USPQ2d 1566 (Fed. Cir. 1990).

Independent claim 54 is directed to a method for preserving a biological material selected from the group consisting of cells, cell aggregates, tissue, organs, biological fluids, natural liposomes and synthetic liposomes, the method comprising: (a) adding a preservation solution to said biological material, the

preservation solution comprising one or more polyphenols; (b) freeze drying the biological material; and (c) storing the biological material under appropriate storing conditions. Claims 55-59 and 63 depend directly from claim 54. Claim 64 has been cancelled without prejudice or disclaimer.

In contrast, Gen is directed to Gen describes the formation of a protein complex with polyphenols, such as catechins for sustained release drug delivery, for introducing genes into cells, etc. The resulting complex may be subjected to freeze drying. See Gen, generally.

However, unlike the presently claimed subject matter, Gen does not teach or suggest a method for preserving a biological material ***selected from the group consisting of cells, cell aggregates, tissue, organs, biological fluids, natural liposomes and synthetic liposomes***, as presently claimed. Therefore, Gen does not teach each and every element of the claimed subject matter.

In view of the foregoing, it is submitted that Gen does not teach, either expressly or inherently, each and every element claimed in claims 54--59 and 63, as required for anticipation under 35 USC § 102 (b). Accordingly, the Examiner is respectfully requested to withdraw this.

**VII. At page 7 of the Official Action, claim 92 has been rejected under 35 USC § 102(b) as being anticipated by Wiggins et al. (US Patent Publication No. 2002/0177116).**

The Examiner asserts that Wiggins et al. teach each and every element of claim 92.

In view of the remarks set forth herein, the rejection of claims 92 is respectfully traversed.

The test for anticipation is whether each and every element as set forth is found, either expressly or inherently described, in a single prior art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987); MPEP § 2131. The identical invention must be shown in as complete detail as is contained in the claim. *Richardson v. Suzuki Motor Co.*, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989); MPEP §2131. The elements must also be arranged as required by the claim. *In re Bond*, 15 USPQ2d 1566 (Fed. Cir. 1990).

Independent claim 92 has been amended to recite A method for preserving a biological material, comprising: (a) adding a preservation solution to the biological material, the preservation solution comprising one or more polyphenols and being essentially free from any polyalcohol; (b) freeze drying the biological material; and (c) storing the biological material under appropriate storing conditions.

In contrast, Wiggins et al. describe compositions and methods for the preservation of biological material. The preservation solution according to Wiggins et al. is to include one or more ions (See Wiggins et al. at paragraph [0013]) so as to be isotonic with the biological material to be preserved (See Wiggins et al. at paragraph [0064]).



However, Applicants submit that nowhere in Wiggins et al. is the use of polyphenols for freeze drying biological material taught. Thus, Wiggins et al. cannot be regarded as anticipating the present invention comprising in the cryopreservation solution at least one polyphenol.

In view of the foregoing, it is submitted that Wiggins et al. do not teach, either expressly or inherently, each and every element claimed in claim 92, as required for anticipation under 35 USC § 102 (b). Accordingly, the Examiner is respectfully requested to withdraw this rejection.

***VIII. At page 7 of the Official Action, claims 54-56, 58-60, 62-64 and 66 have been rejected under 35 USC § 103(a) as being obvious over Mann et al. (US Patent Application Publication No. 2003/0059338).***

The Examiner asserts that it would have been obvious to combine the stabilizers of Mann et al. in the form of solutions with the biological material to be sterilized.

Applicants note that claim 64 has been cancelled without prejudice or disclaimer. Accordingly, the rejection of claim 64 has been obviated.

In view of the foregoing, Applicants respectfully traverse the rejection of claims 54-56, 58-60, 62-63 and 66.

To establish a *prima facie* case of obviousness, the PTO must satisfy three requirements. First, as the U.S. Supreme Court very recently held in *KSR International Co. v. Teleflex Inc. et al.*, 550 U. S. 398 (2007), "a court must ask whether the improvement is more than the predictable use of prior art elements according to their established functions. ...it [may] be necessary for a court to look to interrelated teachings of multiple patents; the effects of demands known

to the design community or present in the marketplace; and the background knowledge possessed by a person having ordinary skill in the art, all in order to determine whether there was an apparent reason to combine the known elements in the fashion claimed by the patent at issue. ...it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does... because inventions in most, if not all, instances rely upon building blocks long since uncovered, and claimed discoveries almost of necessity will be combinations of what, in some sense, is already known." (*KSR*, 550 U.S. at 417). Second, the proposed modification of the prior art must have had a reasonable expectation of success, determined from the vantage point of the skilled artisan at the time the invention was made. *Amgen Inc. v. Chugai Pharm. Co.*, 18 USPQ2d 1016, 1023 (Fed. Cir. 1991). Lastly, the prior art references must teach or suggest all the limitations of the claims. *In re Wilson*, 165 USPQ 494, 496 (C.C.P.A. 1970).

Regarding motivation to modify properly combined references, **MPEP 2143** states that where the prior art conflicts, all teachings must be considered and that the fact that references can be combined or modified is not sufficient to establish *prima facie* obviousness. **MPEP 2143** further states that there must be some suggestion or motivation to modify the references, and there must be a reasonable expectation of success. In addition, the prior art reference or references when properly combined, must teach or suggest all the claim limitations.

**MPEP 2143.01** states that a proposed modification cannot render the prior art unsatisfactory for its intended purpose. If it does, then there is no suggestion or motivation to make the proposed modification. Further, the proposed modification cannot change the principle operation of a reference.

It is submitted that a proper case of *prima facie* obviousness has not been established because there is no motivation to modify Mann et al. to arrive at the presently claimed subject matter and Mann et al. is not enabled for anything more than freezing of pertinacious material, such as monoclonal immunoglobulin, hemophiliac clotting factor VIII, thrombin, etc.

Independent claim 54 is directed to a method for preserving a biological material selected from the group consisting of cells, cell aggregates, tissue, organs, biological fluids, natural liposomes and synthetic liposomes, the method comprising: (a) adding a preservation solution to said biological material, the preservation solution comprising one or more polyphenols; (b) freeze drying the biological material; and (c) storing the biological material under appropriate storing conditions. Claims 55-56, 58-60, 62-63 and 66 all depend, either directly or indirectly, from claim 54.

Briefly, Applicants submit that the presently claimed subject matter recites, for the first time, that the addition of at least one polyphenol to preservation solution increase the viability of the freeze dried cells and maintain their functionally post thawing. Applicants note that the presently claimed subject matter is aimed at preservation of cells or cell containing tissues or organs. In support of this, Applicants note that the present specification, at the paragraph

bridging pages 12 and 13, provides that:

*It was found that the biological material preserved in accordance with the invention can be preserved even if having a volume exceeding 1 ml without any substantial change in its properties, e.g. while maintaining the viability of living matter where such material includes living matter, for periods exceeding about 40 days and even exceeding 60 days. Thus, the present invention provides, by another aspect, a preserved viable biological material, having a volume exceeding 1 ml, preserved for a period exceeding 40 days.*

Further at page 22, the specification provides that:

*Results are shown in Figures 1 and 3, wherein we can see that samples frozen with a solution composed of EGCG and dextran had **higher viability rates** as detected by the Pentra60 machine. In addition, morphological evaluations also showed that RBC frozen with EGCG and dextran had a substantially normal morphological appearance.*

In addition at page 23, the specification provides that:

*We can see that almost 80% of the cells survived when freezing 200ml of RBC suspension. To the inventors best knowledge, **such high viability rate in such large volume**, when freezing a the cells without any intracellular cryoprotectants has been regarded as impossible, until now.*

At page 33, the specification provides that:

*The viability of the cells after freezing and freeze drying with different freezing solutions (as described above) was assayed. Figure 6 shows the viability rates as demonstrated by membrane integrity of the cells. Figure 6 shows that the solution composed of EGCG and trehalose gave **best results** after freeze thawing and freeze drying.*

Finally, at page 34, the specification provides that:

*Figure 7 indicates that viability increased with EGCG concentration after freeze thawing and after freeze drying.*

In addition, Applicants submit herewith Attachment A. Attachment A is a **post filing** paper by the inventors (based on the subject matter of the present application) was shown that the addition of polyphenol, such as EGCG to mononuclear cells (umbilical cord blood cells, UCB) prior to freeze drying improved cells' viability and maintain their cologenic capability, i.e., capability of the stems cells to differentiate, post thawing, into different cells. See Attachment Arav et al., "Freeze-Drying of Mononuclear Cells Derived from Umbilical Cord Blood Followed by Colony Formation," [www.plosone.org](http://www.plosone.org), Volume 4, Issue 4, p e5240 (April 1, 2009), generally.

In contrast to the presently claimed subject matter, Mann et al. describe sterilizing biological materials to reduce the level of one or more active biological contaminants or pathogens, such as viruses, yeasts, molds, fungi, prions or similar agents responsible, alone or in combination, for TSEs and/or single or multicellular parasites. The methods described by Mann et al. use flavonoid/flavonol stabilizers in sterilizing biological materials with irradiation. See Mann et al. at the Abstract.

In addition, Applicants note that there would be no motivation to modify Mann et al. to arrive at the presently claimed subject matter. In this regard, while Mann et al. include cells in its definition of biological material, Mann et al. describe **a method for sterilization by irradiation of the biological material so as to eliminate cellular contaminations**. Thus, Mann et al., in fact, aims at reducing and even eliminating any cellular matter in the material. Therefore, Applicants submit that there is no motivation to modify Mann et al. to use polyphenols for

retaining cell viability following cryopreservation because doing so would destroy the principle of operation described in Mann et al.

Further, while Mann et al. makes a mere mention of “stem cells, islet of Langerhans cells and other cells for transplantation, including genetically altered cells; red blood cells; white blood cells, including monocytes; and platelets,” ***Mann et al. is not enabled for anything more than freezing of pertinacious material, such as monoclonal immunoglobulin, hemophiliac clotting factor VIII, thrombin, etc.*** In this regard, Applicants note that Mann et al. only provide examples of freezing pertinacious material, such as monoclonal immunoglobulin, hemophiliac clotting factor VIII, thrombin, etc, which, as known to those of ordinary skill in the art is very different than the presently claimed subject matter.

As indicated above, there is a great difference between freezing a pertinacious material and freezing cells or cells containing tissues and organs. Further, the viability of the biological material is not even discussed by Mann et al. and, therefore, Applicants submit that high viability could not have been expected when using non-permeating cryoprotectants, such as EGCG. Accordingly, Applicants submit that the presently claimed subject matter is not rendered obvious by Mann et al.

In view of the remarks set forth herein, it is submitted that nothing in any of the applied references, taken alone or together, renders claims 54-56, 58-60, 62-63 and 66 obvious within the meaning of 35 USC § 103 (a). Accordingly, the Examiner is respectfully requested to withdraw this rejection.

**IX. At page 8 of the Official Action, claims 54-61, 63 and 64 have been rejected under 35 USC § 103(a) as being obvious over Anzaghi et al. (WO 03/099040).**

The Examiner asserts that it would have been obvious to combine the stabilizers of Anzaghi et al. in the form of solutions with the biological material to be sterilized.

Applicants note that claim 64 has been cancelled without prejudice or disclaimer. Accordingly, the rejection of claim 64 has been obviated.

In view of the foregoing, Applicants respectfully traverse the rejection of claims 54-61 and 63.

A brief discussion of the relevant authority regarding obviousness is set forth above with regard to the previous rejection. The discussion is incorporated herein by reference.

It is submitted that a proper case of *prima facie* obviousness has not been established because Anzaghi et al. do not teach or suggest all the limitations of the claims as required by *In re Wilson*. In addition, there is no motivation to modify Anzaghi et al. to arrive at the present subject matter.

Independent claim 54 is directed to a method for preserving a biological material selected from the group consisting of cells, cell aggregates, tissue, organs, biological fluids, natural liposomes and synthetic liposomes, the method comprising: (a) adding a preservation solution to said biological material, the preservation solution comprising one or more polyphenols; (b) freeze drying the biological material; and (c) storing the biological material under appropriate storing conditions. Claims 55-61 and 63 all depend, either directly or indirectly,

from claim 54. A detailed discussion of the presently claimed subject matter is presented above with regard to the previous rejection. The discussion of the present subject matter is incorporated herein by reference.

In contrast to the presently claimed subject matter, Anzaghi et al. describe a dietary supplement containing all of the natural components of wine, except for the volatile ones, in particular ethanol. The dietary supplement is suitable for oral administration and contains antioxidant complexes present in wine vinasses combined with one or more bioavailability promoters. The wine vinasse is regarded as waste matter containing essential compounds, such as anti-oxidants complexes and other typically small molecular weight compounds. This, however, does not include viable cells. See Anzaghi et al. at the Abstract.

However, unlike the presently claimed subject matter Anzaghi et al. do not teach or suggest a method for preserving a biological material selected from the group consisting of cells, cell aggregates, tissue, organs, biological fluids, natural liposomes and synthetic liposomes, the method comprising: (a) adding a preservation solution to said biological material, the preservation solution comprising one or more polyphenols; (b) freeze drying the biological material; and (c) storing the biological material under appropriate storing conditions, as presently claimed.

In support of his position, the Examiner indicates that the claims of the present application do not require any particular storing procedures storing times or storing conditions. Regardless, the method of Anzaghi et al. still cannot be employed for freeze drying or cryopreserving viable cells. In this regard



Applicants point to Example 2, which makes use of a temperature of  $-10^{\circ}\text{C}$  during freeze-drying. As well appreciated by those versed in the art, ***such temperature which is above the conventional storing temperature of cells is above the glass transition temperature of solutions that can be used for freezing cells***, particularly for freezing mammalian cells. As such, it will result in fusion and recrystallization and thereby damage the cells. Therefore, there is no motivation to modify the subject matter described in Anzaghi et al. to obtain the presently claimed subject matter.

In addition Applicants submit that freezing of food in the food industry, as described by Anzaghi et al. is not analogous to cryopreserve cells. Therefore, it would not have been obvious from Anzaghi et al. that cryopreserving cells with at least one polyphenol would increase viability and maintain functionality of the post thawed cells.

In view of the remarks set forth herein, it is submitted that nothing in any of the applied references, taken alone or together, renders claims 54-61 and 63 obvious within the meaning of 35 USC § 103 (a). Accordingly, the Examiner is respectfully requested to withdraw this rejection.

**CONCLUSION**

In view of the foregoing, Applicants submit that the application is in condition for immediate allowance. Early notice to that effect is earnestly solicited. The Examiner is invited to contact the undersigned attorney if it is believed that such contact will expedite the prosecution of the application.

In the event this paper is not timely filed, Applicants petition for an appropriate extension of time. Please charge any fee deficiency or credit any overpayment to Deposit Account No. 14-0112.

Respectfully submitted,

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# Freeze-Drying of Mononuclear Cells Derived from Umbilical Cord Blood Followed by Colony Formation

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## Abstract

**Background:** We recently showed that freeze-dried cells stored for 3 years at room temperature can direct embryonic development following cloning. However, viability, as evaluated by membrane integrity of the cells after freeze-drying, was very low; and it was mainly the DNA integrity that was preserved. In the present study, we improved the cells' viability and functionality after freeze-drying.

**Methodology/Principal Findings:** We optimized the conditions of directional freezing, i.e. interface velocity and cell concentration, and we added the antioxidant EGCG to the freezing solution. The study was performed on mononuclear cells (MNCs) derived from human umbilical cord blood. After freeze-drying, we tested the viability, number of CD34<sup>+</sup>-presenting cells and ability of the rehydrated hematopoietic stem cells to differentiate into different blood cells in culture. The viability of the MNCs after freeze-drying and rehydration with pure water was 88%–91%. The total number of CD34<sup>+</sup>-presenting cells and the number of colonies did not change significantly when evaluated before freezing, after freeze-thawing, and after freeze-drying ( $5.4 \times 10^4 \pm 4.7$ ,  $3.49 \times 10^4 \pm 6$  and  $6.31 \times 10^4 \pm 12.27$  cells, respectively, and  $31 \pm 25.15$ ,  $47 \pm 45.8$  and  $23.44 \pm 13.3$  colonies, respectively).

**Conclusions:** This is the first report of nucleated cells which have been dried and then rehydrated with double-distilled water remaining viable, and of hematopoietic stem cells retaining their ability to differentiate into different blood cells.

**Citation:** Natan D, Nagler A, Arav A (2009) Freeze-Drying of Mononuclear Cells Derived from Umbilical Cord Blood Followed by Colony Formation. PLoS ONE 4(4): e5240. doi:10.1371/journal.pone.0005240

**Editor:** Joel M. Schnur, George Mason University, United States of America

**Received:** September 24, 2008; **Accepted:** February 10, 2009; **Published:** April 21, 2009

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**Funding:** Core Dynamics has funded this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript

**Competing Interests:** I am a co-inventor of the following patent applications in the field of UCB cryopreservation, which are licensed to Core Dynamics. WO03/020874 - Improved Method for Freezing Viable Cells · WO07/015252 - Somatic Cells for Use in Cell Therapy · WO05/072523 - Biological Material and Methods and Solutions for Preservation Thereof

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## Introduction

Cryopreservation of hematopoietic stem cells (HSC) is the backbone of clinical stem cell transplantation (SCT), and this technique is an essential part of autologous SCT [1], cord blood transplantation (CBT) and in many cases, allogeneic SCT [2], particularly when the donor is elderly and the stem cells are not readily available. Moreover, in haplo-identical transplants, the donor undergoes multiple stem cell mobilization and harvesting, which necessitates cryopreservation of the graft [3].

Hematopoietic as well as other somatic cells are currently cryopreserved and stored in liquid nitrogen (LN) tanks, in nitrogen vapor phase or in  $-80^{\circ}\text{C}$  freezers [4,5,6]. This conventional mode of cryopreservation is prone to transient warming events and various other hazards, such as cross-contamination [7,8], and results in cell losses of 20 to 30% [9]. In contrast, freeze-drying (i.e. lyophilization) of the cells should theoretically substantially decrease the risks associated with storage at ultra-low temperatures, thus simplifying the procedure, reducing costs and allowing for better management of sample storage and transport. These advantages are especially important when long-term preservation is required, e.g. for potential use at an advanced age. In addition,

freeze-drying does not involve the thawing process to which cell damage is attributed, particularly at large volumes (e.g. from recrystallization).

In order to freeze-dry the cells, we first had to develop a method that would enable freezing in the absence of permeating cryoprotectant agents (CPAs) such as DMSO, glycerol or ethylene glycol, to name a few, and would enable the use of additives that have a high glass transition temperature ( $T_g$ ) and that are solid at room temperature. Overcoming these obstacles is not simple. As Thomas A. Jennings said "most investigators have at times overlooked the importance of the freezing process...while simple in concept, the freezing process will be shown to be perhaps the most complex and least understood step in the lyophilization process" [10]. The major damaging factors associated with freeze-drying liposomes are lipid-phase transition (LPT) and fusion [11]. Cellular membrane LPT is also the mechanism underlying damage that occurs during chilling and it is the main obstacle for successful cryopreservation of many cell types, including sperm [12] and oocytes [13]. However, cryopreservation of cells which are more complex than liposomes has even more damage mechanisms, on top of those to the membrane lipids, such as damage to cells structure and cytoskeleton during freezing [14,15],

damage to lysosomes and mitochondria when cells are dehydrated during the freezing process [16]. cells desiccation causes down-regulation of metabolism, increase intracellular viscosity and salt concentrations, denaturation of proteins and the creation of free radicals [17–20]. Chilling injury can be overcome by stabilizing the membrane phospholipids using disaccharides such as sucrose or trehalose [21]. Other approaches to decreasing the damage associated with LPT have involved changing the lipid composition of the membrane by using liposomes in vitro [22] or dietary additives in vivo [23]. Altering membrane lipid composition has been shown to improve the freeze-drying of platelets [24]. The second factor is membrane fusion, which can occur when the dried cells, maintained in a fluid matrix, come into contact [25]. Liposomes stored above the T<sub>g</sub> have been shown to rapidly fuse and become damaged, and it was therefore concluded that glass transition or vitrification is an important factor in decreasing the chances of fusion upon drying [26]. Vitrification is normally achieved by combining a high concentration of CPA (high-viscosity), a rapid cooling rate and a small volume (i.e. 0.5  $\mu$ l) [27,28]. Obviously, these conditions are not feasible in the freeze-drying of many cell types because of the need to achieve a stable glass matrix without CPAs, at relatively slow cooling rates and large volumes.

We have recently shown that freeze-dried cells can direct embryonic development following cloning [29]. However, those cells had poor viability, showing mainly nuclear integrity. Here we describe the further development of our freeze-drying technology, resulting in improved cell viability and functionality.

We developed a freezing technique in which a thermal gradient is maintained in a conductive material and the sample to be frozen is moved at a controlled velocity through this gradient. After seeding is performed at the edge of the sample, ice crystals start to propagate at a velocity which is correlated to the velocity at which the sample passes through a predetermined thermal gradient. Cooling rate, calculated as thermal gradient (G) multiplied by velocity (V), can be precisely controlled [30,31]. Using this method, we improved the outcome of freezing bulk samples, such as large volumes of sperm [31–33] and whole organs [34,35]. In addition, this method was used by others and found superior to conventional equiaxial freezing [36,37].

Epigallocatechin gallate (EGCG) is a polyphenol found naturally in green tea (*Folium Camelliae*). EGCG is a potent antioxidant, with a wide range of physiological effects, including anticarcinogenic, antibacterial, and antiviral properties [38–40] and, of course, it reduces oxidative stress [41]. Furthermore, we recently found that EGCG also reduces heat stress in mice [42]. We used EGCG as a new additive in our cryopreservation solution. Apparently due to EGCG's interaction with the cell membrane [43] and its antioxidative properties, it stabilized the cells during lyophilization and storage.

In the present study, we evaluated the ability to freeze-dry mononuclear cells (MNCs) derived from human umbilical cord blood (HUCB).

## Materials and Methods

### Blood collection and separation

HUCB units were received from mothers who gave their written consent, under approval of the Helsinki committee—approval no. 1341/01, at the “Chaim Sheba Tel Hashomer” Cord Blood Bank.

MNCs were separated on a Ficoll-Paque density gradient. Briefly, 3 ml of whole blood was carefully layered above 3 ml of Histopaque-1077 (Sigma, St. Louise, MO, USA) and centrifuged

for 30 min at 1000 g (with no break), after which the MNC layer was withdrawn, placed into another test tube with 10 ml of phosphate buffered saline (PBS; free of Ca<sup>2+</sup> and Mg<sup>2+</sup>) and centrifuged for 10 min at 250 g. After this centrifugation, the supernatant was discarded, and a freezing solution at room temperature was added to the cell pellet. The volume of the freezing solution varied, depending on the desired cell concentration (we experimented with concentrations ranging from 1 to 10  $\times 10^6$  cells/ml). A 2.5-ml cell suspension was transferred into a 16-mm-diameter glass test tube (Manara, Kibutz Manara, Israel) which was then frozen in an MTG-1314 apparatus (Core Dynamics, Nes-Ziona, Israel). Samples were placed in the refrigerator for 10–39 minutes before being put in the MTG device for the freezing process.

### Freezing solution

Our cryopreservation solutions were based on Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS supplemented with one or more of the following additives: (1) 0.1 M trehalose (Sigma), (2) 12.5% (w/v) human serum albumin (HSA; Kamada, Beit-Kama, Israel), (3) epigallocatechin gallate (EGCG) (>98% purity; Zhejiang Yixin Pharmaceutical, Lanxi, China), (4) dextran 40 (Pharmacosmos, Holbaek, Denmark). For most of the experiments, we settled on IMT-2 solution, composed of 0.945 mg/ml EGCG and 0.1 M trehalose dissolved in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS.

### Freezing and drying

Freezing was performed in the MTG-1314 apparatus. The freezing-machine temperatures were set to: 5°C to –10°C to –40°C to –70°C. Seeding was performed at the tip of the test tube as the samples entered the cold block (at –10°C). The velocity at which the samples were pushed through the freezing machine was set at 0.2 mm/s, resulting in a calculated cooling rate of 5.1°C/min; the actual cooling rate, as measured by placing thermocouples in a sample, was 4.05°C/min. After completion of the freezing process, samples were stored in a LN tank. Samples were then either thawed or moved on to the drying process which was performed in a commercial lyophilizer (Labconco, Kansas City, MO, USA).

Lyophilization was performed by putting the frozen samples in the commercial lyophilizer near the condenser (Freezone Plus 6, Labconco), which reaches a temperature of –80°C, for 3.5 days. This freeze-drying system is a very simple device, in which neither shelf temperature nor vacuum pressure can be controlled or recorded.

### Thawing, rehydration, and storage

Thawing was performed by immersing the frozen samples in a water bath heated to 37°C. At the end of the lyophilization process, samples were either immediately rehydrated or stored, the latter by putting the samples within an aluminum pouch under vacuum and heat-sealing the pouch using vacuum chamber packaging machine Audionvac VMS 163 (Audion Elektro, Weesp, The Netherlands). The samples were then stored in a refrigerator (2°C–8°C) or at room temperature (25°C) for up to 1 week. Rehydration was performed by adding 2.4 ml double-distilled water (DDW) that had been pre-warmed to 37°C.

After both thawing and rehydration, samples were assessed for viability by Syto-13/PI staining, for CD34<sup>+</sup>-presenting cells by fluorescence-activated cell sorting (FACS) and for colony-forming units (CFU) by assay.

All assessments were performed before and after cryopreservation.

### Residual moisture content and Tg measurements

Three samples from three different donors were evaluated following freeze-drying for their residual moisture content and for glass transition temperature (Tg). For the Tg evaluations, samples were sent to the Analytical Research Services and Instruments unit of Ben-Gurion University where the measurement was performed using a differential scanning calorimeter (DSC) 821e (Mettler, Toledo, OH). The samples were heated from  $-80^{\circ}\text{C}$  to  $50^{\circ}\text{C}$  at  $10\text{ K/min}$ , blank curve corrected, in a nitrogen atmosphere ( $80\text{ cm}^3/\text{min}$ ).

For the residual moisture measurement, we used an HB43 halogen moisture analyzer (Mettler). In brief, samples in powdered form following the lyophilization process are heated up in the moisture analyzer. During the heating process, the analyzer weighs the sample for a pre-determined time, and the measurement is recorded as weight loss of the sample with time.

### Viability and recovery

Cell viability was assessed using the live/dead fluorescent stains Syto-13/PI (Molecular Probes®, Invitrogen Corp., Carlsbad, CA, USA) for membrane integrity. The percentage of viable cells was calculated as follows:

$$\% \text{ Viable cells} = (\text{Live cells after freeze-thawing or freeze-drying} / \text{Live cells before freezing}) \times 100.$$

Cell recovery was evaluated using the automatic cell counter Pentra 60 (Horiba ABX, Montpellier, France). Percent cell recovery was determined as follows:

$$\% \text{ Recovery} = (\text{Cell concentration after freeze-thawing or freeze-drying} / \text{Cell concentration before freezing}) \times 100.$$

### Surface antigens

We examined the MNCs from each HUCB unit before and after freeze-thawing or freeze-drying for number of cells presenting CD34<sup>+</sup> antigen. Briefly,  $1 \times 10^6$  MNCs were stained with  $1\text{ }\mu\text{g}$  fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies for 20 min at  $4^{\circ}\text{C}$  and then washed in PBS containing  $0.02\%$  (w/v) azide and  $1\%$  (v/v) bovine serum albumin as described previously [44]. The cells were stored at  $4^{\circ}\text{C}$  in  $0.5\text{ ml}$  of  $1\%$  (v/v) paraformaldehyde until analysis. Fluorescence intensity was measured on a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).

### Colony-forming unit assay

CFU assay was performed on MNCs derived from each HUCB unit using fresh samples (i.e. samples exposed to the IMT-2 solution but not frozen) and samples after freeze-thawing or freeze-drying.

We used the protocol and Methocult media from Stem Cells Technologies Inc. (Vancouver, Canada). Briefly, cells were inoculated into methylcellulose medium and incubated in  $5\%$   $\text{CO}_2$  for 14 days. After incubation, the plate was viewed under an inverted microscope, and cell colonies were counted and identified as erythrocytic (CFU-E, BFU-E), granulocytic (CFU-GM), or mixed (CFU-GEMM).

### Scanning electron microscopy

For evaluation of the morphology of dry samples Scanning Electron Microscopy (SEM) was used. MNC derived from HUCB

were frozen in three different solutions: (1) trehalose solution –  $0.1\text{ M}$  trehalose in PBS (without  $\text{Ca}^{+2}$  &  $\text{Mg}^{+2}$ ), (2) EGCG solution –  $0.945\text{ mg/ml}$  EGCG dissolved in PBS (without  $\text{Ca}^{+2}$  &  $\text{Mg}^{+2}$ ), (3) IMT-2. The samples were frozen and dried as described above. After lyophilization has finished samples were gold plated before being placed in the SEM. The voltage of the electron scatter was  $25\text{ kv}$ . In this test the electrons hit the sample and are returned to a detector. We captured a 3D picture on a CRT screen.

### Statistical analysis

At least three replications were performed for each experiment (i.e., using blood from three different donors). At least 300 cells were counted per sample. Means were calculated and differences between treatments were examined by t-tests using the General Linear Model procedure of JMP (SAS Institute, 1994, Cary, NC). Significance was  $P < 0.05$  unless otherwise stated. In all figures, different letters represent statistically different samples. Results are reported as mean  $\pm$  SE.

## Results

### Moisture content and Tg

After freeze-drying, samples were taken out of the lyophilizer and analyzed in the HB43 halogen moisture analyzer. The results showed a residual moisture content of  $4.69 \pm 0.07\%$  in all samples tested (all samples were frozen in IMT-2 solution). The Tg of the dried samples was between  $8.49^{\circ}\text{C}$  and  $25.94^{\circ}\text{C}$ , with a midpoint at  $11.84^{\circ}\text{C}$ .

### Effect of ice interface velocity on survival after freeze-drying

We evaluated the survival of HUCB MNCs after freeze-thawing and freeze-drying at different cooling rates, resulting from different ice interface velocities (V). The freezing solution consisted of  $12.5\%$  (w/v) HSA and  $0.1\text{ M}$  trehalose in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS.

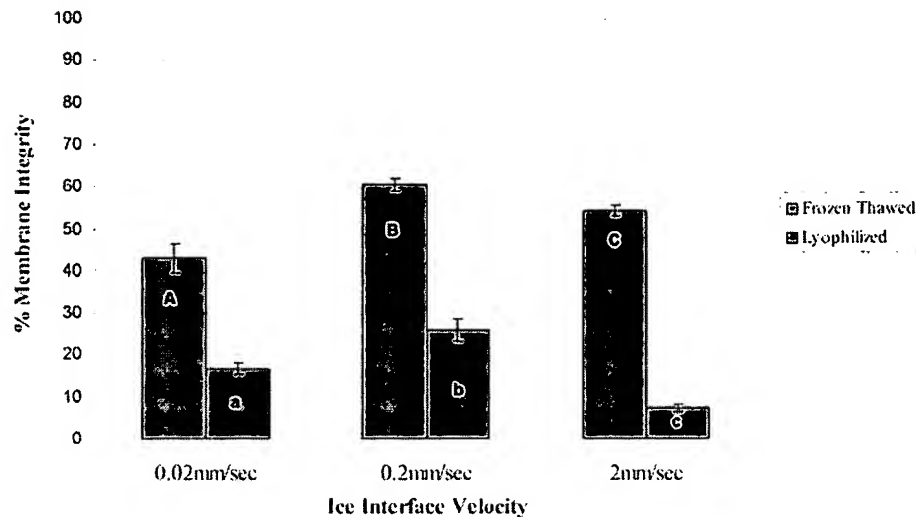
The freezing temperatures in the MTG-1314 were set as described in Materials and Methods. The different cooling rates were achieved by varying the velocity at which the samples were pushed through the predetermined temperatures. The ice interface velocities were:  $0.02$ ,  $0.2$  and  $2\text{ mm/s}$ , resulting in calculated cooling rates of  $0.51$ ,  $5.1$  and  $51^{\circ}\text{C/min}$ , respectively (cooling rate =  $G \times V$ ).

Figure 1 depicts the effect of ice interface velocity on cell survival, according to fluorescent Syto/PI staining after freeze-thawing and freeze-drying. In both cases, the highest viability rates were obtained at the intermediate velocity of  $0.2\text{ mm/s}$ , resulting in  $60\%$  and  $25\%$  of the cells having intact membranes after freeze-thawing and freeze-drying, respectively.

### Effect of different additives on cell survival after freeze-drying

We evaluated the survival of the MNCs after freeze-thawing and freeze-drying in freezing solutions with different viscosities. All other experimental steps were performed as described in Materials and Methods.

Figure 2 presents the effects of different cryopreservation solutions on HUCB-derived MNC viability after freeze-thawing and freeze-drying. The original solution used for the preliminary studies, composed of HSA and trehalose (solution #1), produced rather low viability rates, especially after freeze-drying (only  $10\%$ ). However, the solution consisting of trehalose and EGCG (solution #4) and that consisting of EGCG and dextran (solution #5)



**Figure 1. Percentage membrane integrity after freezing at different ice interface velocities.** The solution used in this experiment was 12.5% (w/v) HSA and 0.1 M trehalose in PBS ( $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free). This experiment was performed on HUCB units from three different donors, in triplicate ( $n=54$ ). Data are presented as mean  $\pm$  SE. doi:10.1371/journal.pone.0005240.g001

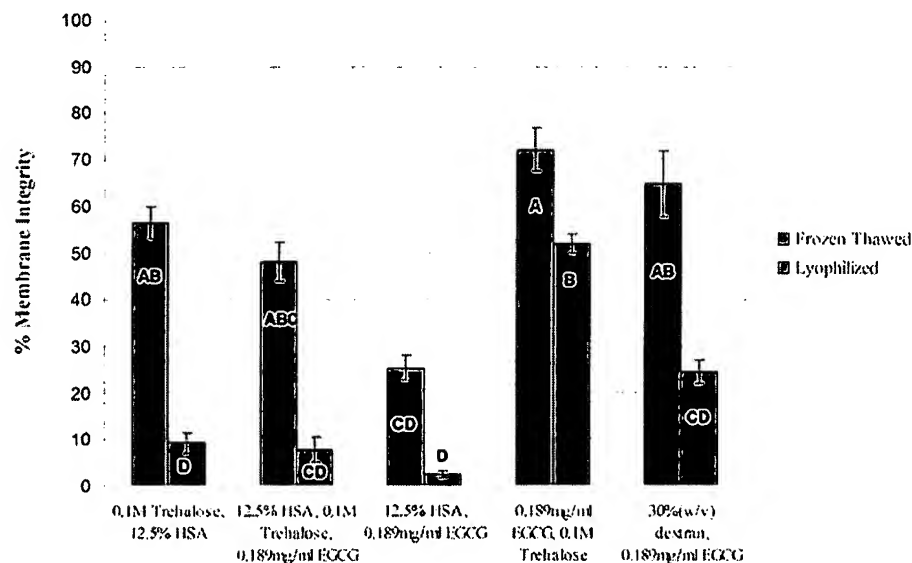
produced higher viability rates, resulting in 70% and 52% of the cells having intact membranes after freeze-thawing and freeze-drying, respectively, for solution #4, and 65% and 25% of the cells having intact membranes after freeze-thawing and freeze-drying, respectively, for solution #5.

We compared cell recovery in the three solutions that gave the highest viability rates. The HSA-trehalose solution yielded recovery rates of 100% and 85% after freeze-thawing and freeze-drying, respectively. However, this solution showed a low viability rate after freeze-drying (10% membrane integrity, see Figure 2). The dextran-trehalose solution yielded recovery rates of

98% and 62% after freeze-thawing and freeze-drying, respectively. This solution showed a rather large reduction in cell recovery after freeze-drying. Combined with a low viability of 25%, only 15% of the cells were actually viable. The trehalose-EGCG solution resulted in 100% recovery after both freeze-thawing and freeze-drying.

#### Dose response of EGCG

We evaluated the effect of different EGCG concentrations on HUCB-derived MNC survival following freeze-thawing and freeze-drying.



**Figure 2. Percentage membrane integrity after freeze-thawing and freeze-drying in different solutions.** All solutions are based on PBS ( $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free). This experiment was performed on HUCB units from three different donors, in duplicate ( $n=60$ ). Data are presented as mean  $\pm$  SE. doi:10.1371/journal.pone.0005240.g002

The freezing solutions were composed of 0.1 M trehalose and EGCG at the following concentrations: 0.189, 0.47, 0.945 or 1.89 mg/ml, all in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS. Results are presented in Figure 3. The highest concentration of EGCG gave the best viability rates after freeze-thawing and freeze-drying, with 97.9% and 88% of the cells having intact membranes, respectively. However, antioxidants are known to have a damaging effect at high doses. Therefore, we used the lower dose of 0.945 mg EGCG/ml (which, with 0.1 M trehalose in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS, makes up IMT-2) in further experiments, as this dose also resulted in very high viabilities—82.8% and 77% of the cells with an intact membrane after freeze-thawing and freeze-drying, respectively. The recoveries in this experiment were more than 98% in all samples, therefore, only viability data is shown since we remained with the same cell number after freeze thawing and freeze drying.

#### Effect of each freezing solution components on post-thaw and post-rehydration viability and the influence of a washing process after these procedures

In this set of experiments we wanted to find out how each of the ingredients composing IMT-2 freezing solution (i.e. EGCG and trehalose) affect the post thaw and post rehydration viability of MNC derived from HUCB. In addition, we also wanted to find out how a washing procedure after thawing and rehydration will affect the viability.

These experiments were done on UCB from 4 different donors. The MNC were suspended with one of the following freezing solutions:

- 1) IMT-2
- 2) PBS (without  $\text{Ca}^{++}$  &  $\text{Mg}^{++}$ ) supplemented with 0.945 mg/ml EGCG.
- 3) PBS (without  $\text{Ca}^{++}$  &  $\text{Mg}^{++}$ ) supplemented with 0.1 M Trehalose

From each unit a total of 12 samples were frozen using the MTG as described above, half of the samples ( $n = 6$ ) continued to the drying process. The experiment was performed with duplicates for each solution. Rehydration and thawing were performed as

described above. Immediately after thawing and rehydration a samples was taken to determine cells concentration and viability, the rest of each sample (1.5 ml) underwent a washing process by centrifuging it for 10 minutes at 250 g after which the supernatant was discarded and the pellets were re-suspended with 1.5 ml RPMI-1640 (Sigma, St. Louise, MO, USA).

In this experiment we calculated the viability after each procedure according to the following formula:

$$\% \text{ Viability} = \left\{ \frac{[\% \text{ viable cells after freeze thawing} \times \text{cell number after freeze drying}]}{[\% \text{ viable cells before freezing} \times \text{cell number before freezing}]} \right\} \times 100$$

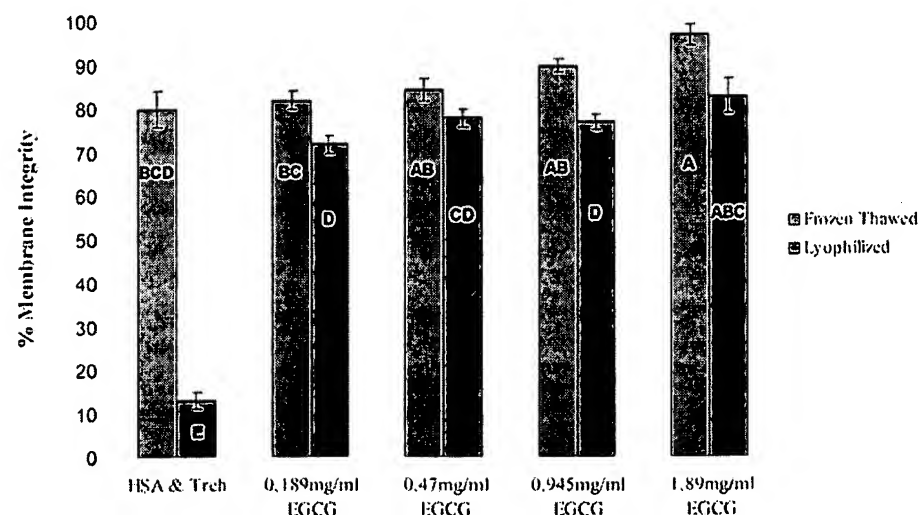
The results after freeze thawing were as follows: for IMT-2 solution  $98.01\% \pm 3.1\%$  immediately after thawing and  $82.75\% \pm 3.1\%$  after washing; for the EGCG solution viability after thawing was  $49.27\% \pm 2.9\%$  and after washing it was  $42.47\% \pm 2.9\%$ ; the EGCG solution resulted with a viability of  $30.1\% \pm 2.9\%$  after freeze thawing and  $19.18\% \pm 2.9\%$  after thawing and washing (Figure 4).

The results after freeze drying and rehydration were as follows: for IMT-2 solution  $91.6\% \pm 2.6\%$  immediately after rehydration and  $64.55\% \pm 3.1\%$  after washing; for the EGCG solution viability after rehydration was  $44.8\% \pm 2.7\%$  and after washing it was  $33.38\% \pm 3.1\%$ ; the EGCG solution resulted with a viability of  $18.52\% \pm 2.6\%$  after rehydration and  $10.28\% \pm 3.1\%$  after washing (Figure 5). The data is presented as mean  $\pm$  SE, ( $n = 48$  for each cryopreservation procedure).

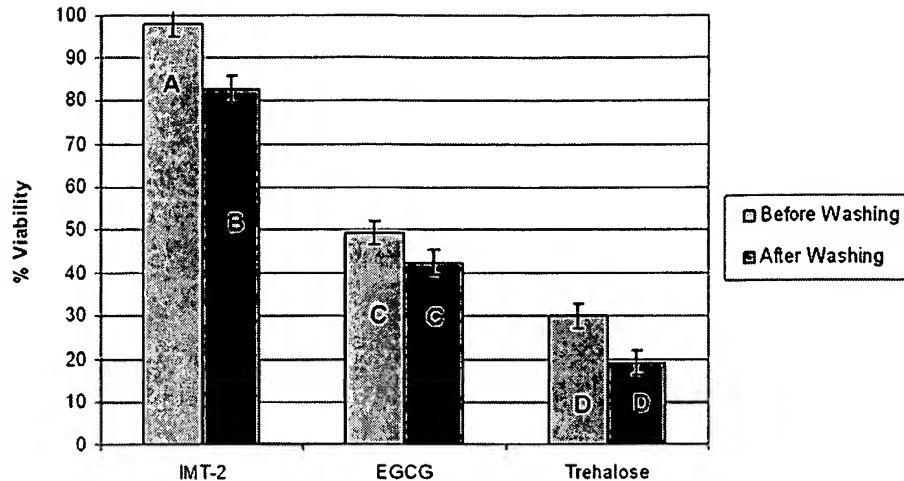
We can see that EGCG has a higher contribution to the post thaw and post rehydration viability of the cells than Trehalose (Figures 4 and 5). Centrifugation has resulted with a reduction in cell viability but only with IMT-2 solution the differences were statistically significant (Figures 4 and 5).

#### Effect of different cell concentrations on their survival after freeze-drying

We evaluated whether cell concentration affects post-lyophilization viability. We tested the following cell concentrations:  $2.5 \times 10^6 \pm 0.4 \times 10^6$  cells/ml,  $5 \times 10^6 \pm 0.3 \times 10^6$  cells/ml and



**Figure 3. Percentage membrane integrity after freeze-thawing and freeze-drying with different concentrations of EGCG.** This experiment was performed on HUCB units from three different donors, in duplicate ( $n = 60$ ). Data are presented as mean  $\pm$  SE. doi:10.1371/journal.pone.0005240.g003



**Figure 4. Viability percentage of MNC derived from HUCB after freeze thawing with one of the following solutions: IMT-2, EGCG solution, Trehalose solution.** The samples were either immediately evaluated or underwent a washing process and then evaluated again. The data is presented as the mean  $\pm$  SE, different letters represent statistical differences between groups ( $p < 0.05$ ). The means were calculated as the number of viable cells after thawing divided by the number of viable cells before freezing. (N=48). doi:10.1371/journal.pone.0005240.g004

$8 \times 10^6 \pm 0.2 \times 10^6$  cells/ml. All samples were frozen in IMT-2 solution, and freezing and drying were performed as described in Materials and Methods. This experiment was performed on HUCB units from three different donors, in triplicate ( $n = 18$ ). Membrane integrity as assayed by Syto/PI staining was as follows:  $69.28 \pm 17.1\%$ ,  $57.01 \pm 14.1\%$  and  $55.9 \pm 5.3\%$  for the cell concentrations listed above, respectively. Thus there was a reduction in viability with increasing cell concentration; however, the differences were not statistically significant.

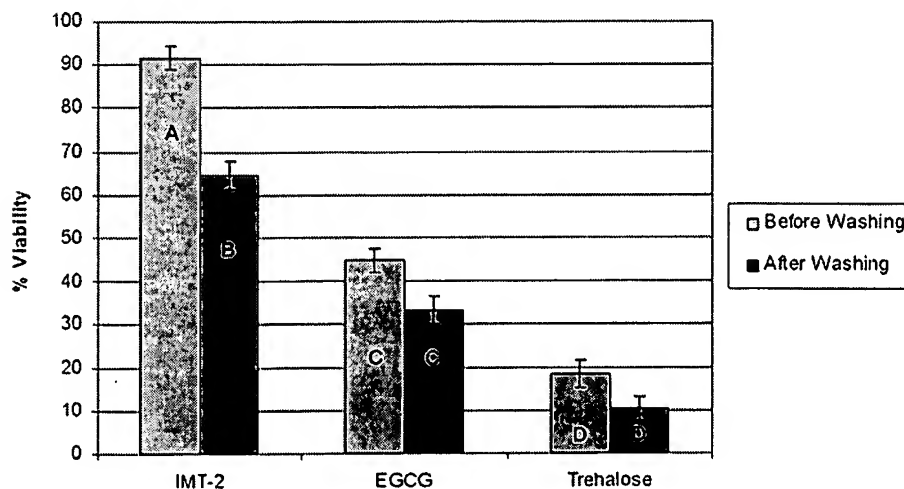
and their viability evaluated by Syto/PI staining. All samples were stored under vacuum and protected from the light. The results were as follows: after 3 days of storage at  $4^\circ\text{C}$ , membrane integrity was  $52.5 \pm 12\%$  and after 1 week at  $4^\circ\text{C}$  it was  $72.1 \pm 11.5\%$ . After 3 days of storage at room temperature, membrane integrity was  $50 \pm 10\%$  and after 1 week at room temperature it was  $50.5 \pm 10\%$ . This experiment was performed on HUCB units from three different donors, in triplicate ( $n = 36$ ). There was no statistical difference between the tested groups.

#### Effect of storage conditions on cell viability

In these experiments, freeze-dried samples of HUCB-derived MNCs were stored in the dry state at two temperatures:  $4^\circ\text{C}$  (refrigeration) and room temperature. Samples were stored for 3 days and for 1 week, after which they were rehydrated with DDW

#### Number of CD34<sup>+</sup> presenting cells before and after freeze-thawing and freeze-drying

In these experiments, MNCs that had been frozen in IMT-2 solution were evaluated for CD34<sup>+</sup> cells before and after freeze-



**Figure 5. Viability percentage of MNC derived from HUCB after freeze drying with one of the following solutions: IMT-2, EGCG solution, Trehalose solution.** The samples were either immediately evaluated or underwent a washing process and then evaluated again. The data is presented as the mean  $\pm$  SE, different letters represent statistical differences between groups ( $p < 0.05$ ). The means were calculated as the number of viable cells after thawing divided by the number of viable cells before freezing. (N=48). doi:10.1371/journal.pone.0005240.g005



thawing and freeze-drying. From each unit of HUCB, at least four test tubes were frozen and subjected to either thawing or drying and rehydration. Cell counts are presented in Table 1.

### Colony-forming unit assay

In these experiments, the same MNC samples used for CD34<sup>+</sup> cell detection were subjected to CFU assay before and after freeze-thawing or freeze-drying and rehydration. The colonies were evaluated and the total number of colonies for each unit is shown in Table 2.

### Discussion

The freeze-drying of cells can be divided into two steps: the first is the freezing process during which large ice crystals are formed, pushing the cells into an area defined as the unfrozen fraction. The unfrozen fraction incorporates the cells that have dehydrated and vitrified in an amorphous matrix during the freezing process. The second step is sublimation of the ice crystals, which occurs in two stages, termed primary and secondary drying. Two requirements for cell survival during freeze-drying are stabilization of the membrane during the drying process [11] and the ability to form a stable vitrified matrix of cells in the unfrozen fraction. Stabilization of the membrane during dehydration is important for both the freeze-drying process and for room-temperature storage of the dried material [25]. Crowe et al. [45,46] have shown that both of these requirements can be fulfilled by using the non-reducing disaccharide trehalose, which is present in many organisms (at up to 20% of their dry weight) that dehydrate almost completely in nature. Intracellular trehalose has been shown to be necessary for successful stabilization of the membrane during freeze-drying of liposomes and cells [47]. In the present study, we show that optimizing the freezing process and providing extracellular trehalose and EGCG is sufficient for successful freeze-drying of MNCs.

When we evaluated the effect of ice interface velocity (Figure 1) on the viability of freeze-thawed and freeze-dried HUCB-derived MNCs, we obtained an inverted U-shaped viability curve in which the highest survival rate was achieved at the intermediate velocity of 0.2 mm/s. We used an MTG-1314 freezing apparatus, which is

**Table 1.** Number of CD34<sup>+</sup> cells before freezing and after thawing and lyophilization.

Exp #	1	2	3
Fresh ( $\times 10^4$ cells)			
Group 1	10.4	1.05	4.85
Mean $\pm$ SD	5.4 $\pm$ 4.7		
After thawing ( $\times 10^4$ cells)			
Group 1	1.9	15.6	0.7
Group 2	2.3	0.25	0.2
Mean $\pm$ SD	3.5 $\pm$ 6		
After rehydration ( $\times 10^4$ cells)			
Group 1	1.6	36.4	1.7
Group 2	----	0.2	2.9
Group 3	5.7	2	0.15
Mean $\pm$ SD	6.3 $\pm$ 12.3		

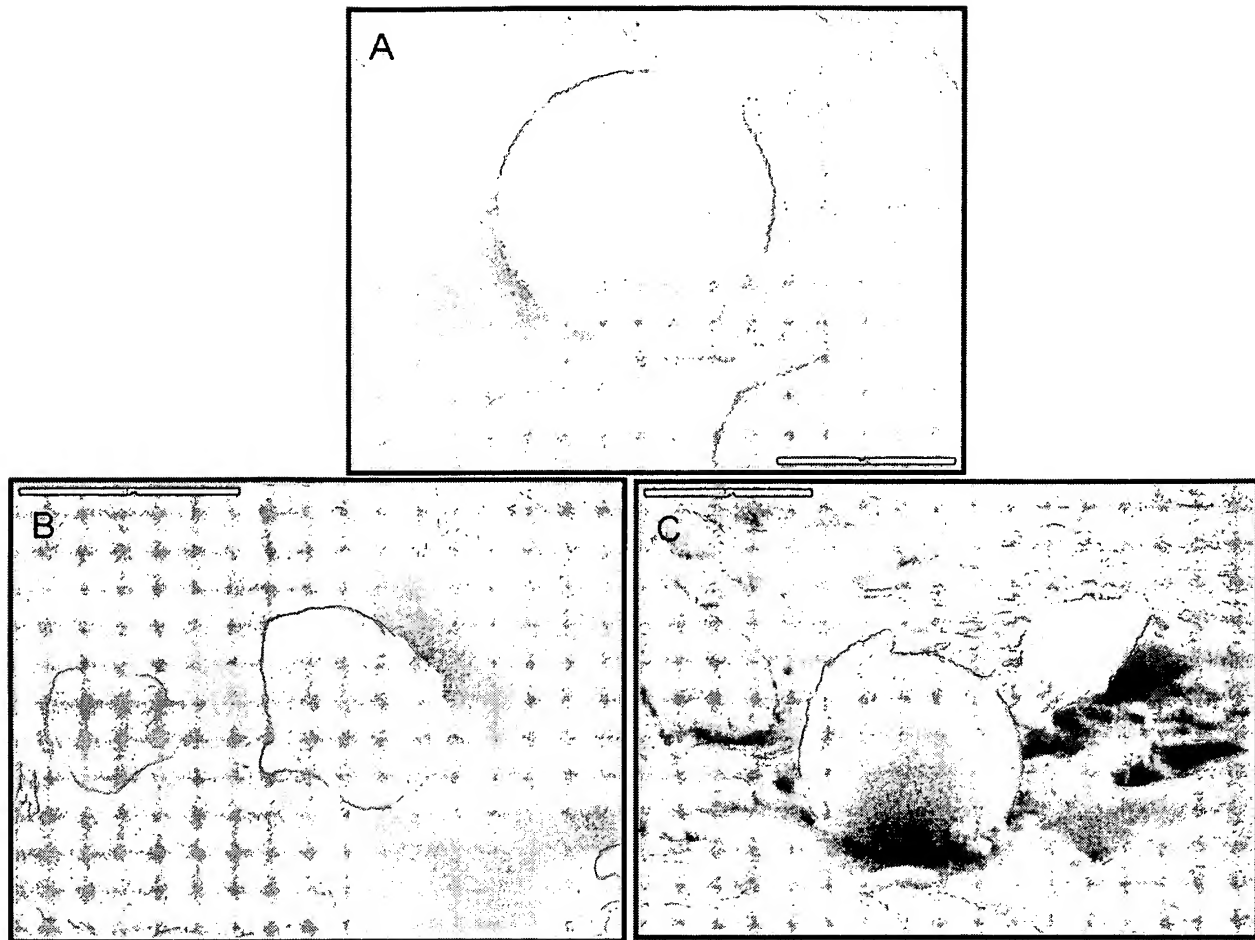
Results are from three different experiments (i.e. different donors); each individual number represents a different sample.  
doi:10.1371/journal.pone.0005240.t001

**Table 2.** The number of colonies grown, before freezing and after thawing and rehydration.

Exp #	1	2	3
<b>Fresh</b>			
Group 1	60 (33-E/BFU; 27-GM)	18 (6-E/BFU; 12-GM)	15 (5-E/BFU; 10-GM)
Mean±SD	31±25.15		
<b>After thawing</b>			
Group 1	12 (4-E/BFU; 8-GM)	21 (3-E/BFU; 18-GM)	10 (GM)
Group 2	53 (31-E/BFU; 22-GM)	29 (8-E/BFU; 21-GM)	108 (1-E/BFU; 107-GM)
Mean±SD	38.83±37.3		
<b>After rehydration</b>			
Group 1	9 (2-E/BFU; 7-GM)	42 (11-E/BFU; 31-GM)	27 (3-E/BFU; 24-GM)
Group 2	30 (1-E/BFU; 29-GM)	28 (1-E/BFU; 27-GM)	12 (GM)
Group 3	13 (1-E/BFU; 12-GM)	43 (4-E/BFU; 39-GM)	6 (GM)
Mean±SD	23.3±13.9		

Results are from three different experiments (i.e. different donors); each individual number represents a different sample and the colony type is given in brackets (erythrocytic- E/BFU; granulocytic - GM).  
doi:10.1371/journal.pone.0005240.t002

based on directional freezing. With the MTG, cooling rate depends on the ice interface velocity (V) and the thermal gradient (G). Interface velocity is known to affect the survival of cells after freeze-drying via its control of the size and morphology of the ice crystals [30]. At a slow ice interface velocity (0.02 mm/s), large ice crystals are formed, minimizing the size of the unfrozen fraction, and thus causing mechanical damage to the cells and cell-shearing. On the other hand, at the high ice interface velocity (2 mm/s), the size of the unfrozen fraction is large but the likelihood of intracellular ice formation increases, since water has less time to diffuse out of the cells during the freezing process [48]. Further examination indicated that our original freezing solution (composed of HSA and trehalose) was not optimal, and that the solution that included EGCG and trehalose was better suited to our purposes (Figure 2). EGCG is an antioxidant which is also known to connect to the polar head groups of membrane lipids [43] and recently it has been shown that EGCG penetrate suspended cells in a time dependant manner [50]. We hypothesized that this ability of EGCG stabilized the membrane during the freeze-thawing and freeze-drying processes; this stabilization was effected in a dose-related manner (Figure 3). This was also shown in the experiments where we have evaluated the effect of each solution additive on the post thaw and post rehydration viability. The results in Figures 4 and 5 demonstrate that the effect EGCG has on the viability of cells is higher than that of Trehalose and that having both of the components in the solution (i.e. IMT-2) has resulted with very high viabilities after freeze



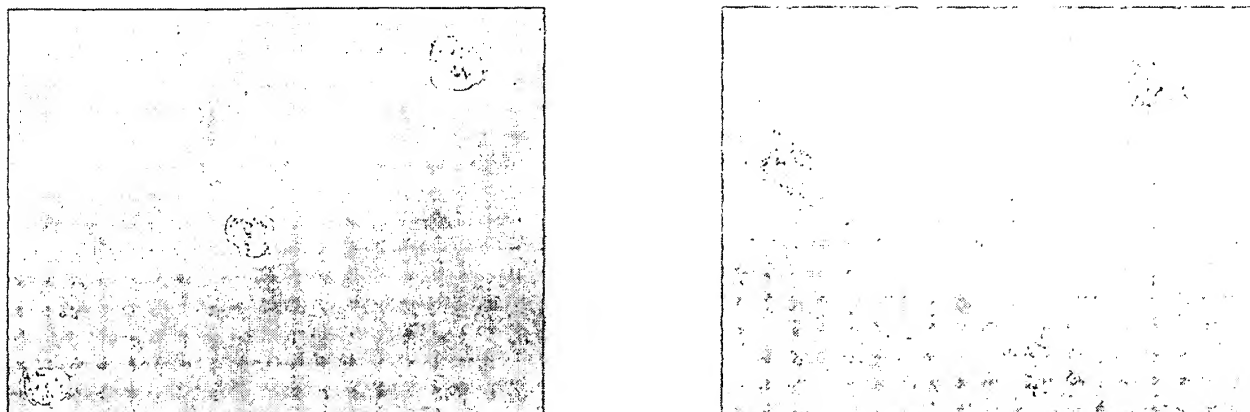
**Figure 6. Pictures of taken using a scanning electron microscope of freeze-dried MNC derived from HUCB.** The pictures where of samples frozen with one of the following solutions: (A) Cells that were frozen with 0.1 M trehalose in PBS (sans calcium & magnesium). (B) Cells that were frozen with 0.945 mg/ml EGCG in PBS (sans calcium & magnesium). (C) Cells that were frozen with IMT-2 solution. Pictures Magnification  $\times 7500$ . doi:10.1371/journal.pone.0005240.g006

thawing and freeze drying. In addition, the viabilities received after freeze drying for IMT-2 and EGCG solution were similar to the viabilities received with these solutions after freeze thawing. Whereas with trehalose there was a much lower viability after freeze drying then after freeze thawing. In our method we do not introduce trehalose into the cells. It has been described that the having intracellular trehalose is of importance in stabilizing membranes for freeze drying [47]. The EGCG ingredient which results with similar viabilities after both procedures imposes sufficient protection on the cells during both freeze thawing and freeze drying. In addition, it seems that both supplements have a synergetic effect since the viability obtained with IMT-2 solution is higher then the sum of each component combined. It has been demonstrated that a stress protein acts synergistically with trehalose to confer desiccation tolerance [49].

As for the effect washing procedure has on the viability; we can see that washing has resulted with a reduction in viability with the IMT-2 solution both after freeze thawing and both after freeze drying, indicating that although immediate viability was very high ( $>90\%$  for each procedure) there was a sub-population of cells which were further damaged by the washing procedure. The viability after washing has decreased more after freeze-drying then after freeze thawing, indicating a larger number of cells that were

impaired although at first passed as viable cells according to live/dead assay (Figures 4 and 5). When looking at the SEM pictures (Figure 6) we can see that cells that were freeze-dried trehalose solution (Figure 6A) had a good morphology similar to what is seen in cells freeze-dried with IMT-2 solution (Figure 6C). Whereas, the cells freeze-dried with EGCG solution (Figure 6B) were less round and shrunk. When observing the viability results after freeze-drying that each solution gave (Figure 5) EGCG solution had a higher viability percentage then that received with the trehalose solution. This strengthens the idea that trehalose needs to be intracellular as well in order to provide its lyo-protective properties. These experiments indicated the importance of both the size of the unfrozen fraction and membrane stabilization.

Stability of the unfrozen fraction is important during the drying process: if the sublimation temperature is above the  $T_g$  of the sample, damage will be incurred due to recrystallization [51]. The cooling rate affects freezing and drying in opposite ways: while it is important that the cooling rate be rapid enough to prevent long exposure to high concentrations of solutes which can cause chemical toxicity and osmotic stress, it should also be slow enough to minimize the chances of intracellular crystallization during freezing. A slow cooling rate is also important for the subsequent drying step, since it allows the growth of large ice crystals, which



**Figure 7. Photographs showing colonies formed after lyophilization and rehydration with DDW of samples frozen in IMT-2 solution.**

doi:10.1371/journal.pone.0005240.g007

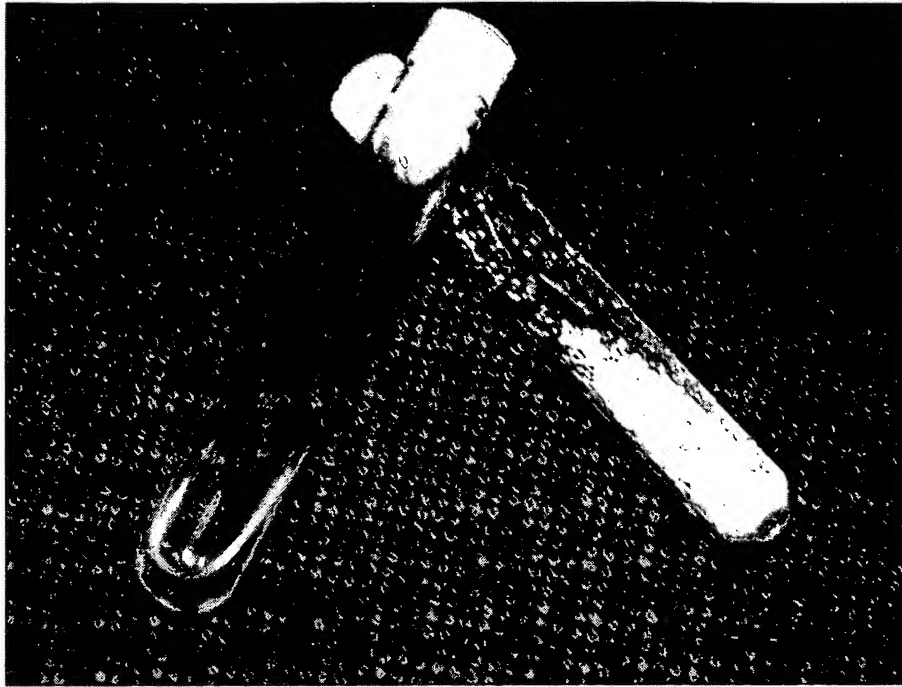
facilitates sublimation during primary drying [52,53]. In the freeze-drying process, vitrification (also termed eutectic solidification) occurs in the unfrozen fraction between the ice crystals which are sublimated in an endothermic process. However, Mazur et al. [54] have shown that decreasing the unfrozen fraction can damage the cells, probably due to mechanical damage by the ice crystals. Therefore, the unfrozen fraction should be small enough to allow vitrification but large enough to reduce the damaging effect of the ice crystals. We believe that directional freezing can combine the optimal conditions needed to achieve large unfrozen fractions for a given concentration of cells and additives, because the size of the ice crystals is determined by the interface velocity [30,31,55] and is precisely controlled by the rate of movement through the thermal gradient.

It is important that the additive(s) have a high  $T_g$ . Moreover, the concentration of the additive is positively correlated with its  $T_g$  [56]. Increased solution viscosity will increase the chances of vitrification because at  $10^{14}$  poise, the solution forms a glass [27,28]. In the present work, we used trehalose as the additive as it has a high  $T_g$  combined with membrane-stabilizing properties [57]. We believe that EGCG also gave better results (Figures 2, 3, 4, and 5) than other additives because of its ability to interact with the cell's membrane [43], together with its antioxidant properties [58,59]. Cell concentration is an important factor during both freezing [60] and drying. Crowe et al. [11] have shown that the primary role of vitrification is to prevent the cells from coming in close proximity in the dry state, thereby preventing fusion and cell damage. Therefore, it can be concluded that cell concentration, unfrozen fraction size and vitrification are important parameters for both the frozen and the dried states, a conclusion that was confirmed in the present study.

The experiments performed with different concentrations of MNCs showed a decrease in viability with increasing cell concentration. A similar observation has been made with erythrocytes and hepatocytes [61]. There are three ways in which cell concentration can influence survival after freeze-drying: (a) During the freezing process, the higher the cell concentration, the higher the chances that more cells will be subjected to mechanical damage (i.e. shearing damage) [60]. (b) During drying, the chances of cell fusion increase with cell concentration. This is because of the increased likelihood of cell membranes coming into contact with each other and fusing, and because a high concentration of cells reduces the  $T_g$ . (c) The freezing solution is composed of a

certain concentration of additives. When the number of cells exposed to this concentration varies, so does the additive's ability to provide cryoprotection (e.g. by stabilize the membrane). The higher the cells concentration in a pre-fixed volume the fewer individual cells are exposed to the additives in the solution. In the present report, we used IMT-2, made up of trehalose and EGCG, a composition which stabilized the membrane and prevented both fusion and oxidative stress [41,43,47].

When we performed the CFU assay on freeze-thawed and freeze-dried and rehydrated MNCs, we observed various types of colonies (granulocytes, macrophages and erythrocytes) after 14 days of culture, indicating, for the first time, that HSCs which have been dried and rehydrated with pure DDW retain their functionality and are able to form colonies of both myeloid and erythroid lineages (Table 2). In addition, we show that after freeze-thawing and freeze-drying and rehydration, cells also retain their CD34<sup>+</sup> surface antigen (Table 1), a marker for hematopoietic progenitor cells. However, with both the CD34<sup>+</sup> and colony-formation data, we noted a large variation among samples taken from the same donor and subjected to the same conditions. Although we do not have any explanation for this variation, it has been reported by others as well [62]. In addition, EGCG may have an effect on the clonogenic capacity of the cells. Several effects of EGCG on T cells have been reported, including inhibition of T-cell proliferation and inhibited division of stimulated T cells [63]. In addition, it has been found that EGCG binds to CD4<sup>+</sup> T cells and it has even been suggested as a way of preventing HIV infection [64]. EGCG has also been found to bind to CD11b and CD8<sup>+</sup> T cells and to exert strong suppression of CD8<sup>+</sup> cell migration and adhesion [65]. We theorize that the same mechanism via which EGCG stabilizes cell membranes during freeze-drying is also responsible for the cells' decreased ability to proliferate and differentiate normally via an inhibitory effect. This was evidenced by the number and morphology of the colonies before freezing (and after adding IMT-2 solution) as well as after freeze-drying and rehydration, which yielded a morphology similar to that seen in Figure 7. Nevertheless, certain possibilities for minimizing this damaging effect should be further evaluated, such as removing residual EGCG from the solution by centrifugation after thawing or rehydration, or diluting the EGCG concentration by adding solution—indeed, washing the cells after rehydration increased cell proliferation (data not shown). The effect of EGCG on the cells might be stronger in vitro than when



**Figure 8.** A photo of dry MNCs derived from HUCB after freeze-drying, before (right) and after (left) rehydration with DDW.  
doi:10.1371/journal.pone.0005240.g008

administered *in vivo*. We have performed experiments with this solution on MNCs derived from male mouse bone marrow which we then transfused into sub-lethally irradiated female mice: in that experiment, we saw an increase in mouse survival. One month after the transfusion, PCR analysis of the transfused mice's blood revealed the presence of the Y chromosome. This work, performed at Hadassah Ein-Kerem Hospital (data not shown), suggested that the cells were capable of incorporation into the bone marrow and of forming new white blood cells. It may be that EGCG binding to the cells is reversed *in vivo* at the concentration used in the IMT-2 solution. This hypothesis warrants further investigation.

We believe that the combination of IMT-2 solution and optimal velocity of ice propagation using directional freezing results in the reduced damage observed here at relatively slow cooling rates and in the absence of CPAs. The reason for the higher viability of cells after freezing relative to post-freeze drying is probably associated with the non-optimal conditions of the drying or storage process. Rindler et al. [66] have shown the importance of sublimation temperature, as recrystallization occurs at a sublimation temperature that can be damaging to the cells. Here we found that maintaining the cells at a temperature of  $-35^{\circ}\text{C}$  leads to cell damage and to a loss of membrane integrity (data not shown). In addition, storage at low temperature ( $4^{\circ}\text{C}$ ) was found to be superior to storage at room temperature. Figure 8 shows photographs of samples after freeze-drying and after rehydration. The sample resembles a powder after freeze-drying (Figure 8), with

its relatively low residual moisture content of  $4.69 \pm 0.07\%$ . At this moisture level, the Tg midpoint was  $11.8^{\circ}\text{C}$ , indicating that the storage temperature needs to be lower than that to maintain cells under the Tg. The results of the Tg measurements performed on the dry samples, showing a Tg midpoint at  $11.84^{\circ}\text{C}$  (ranging from  $8.49^{\circ}\text{C}$  to  $25.94^{\circ}\text{C}$ ). The Tg values found here are relatively low compared to observation of Crowe et al [67]. We speculate that the samples may have obtained moisture prior to the Tg measurements. We currently evaluate ways to improve these results. Therefore, with the residual moisture content found here, the samples can be stored under refrigeration (i.e.  $2^{\circ}\text{C}$ – $8^{\circ}\text{C}$ ), but not at room temperature.

We are currently working on determining the optimal conditions for sublimation and storage which we believe will result in further increasing the survival of cells after freeze-drying. In addition, we are up-scaling the MTG freezing device to allow for large-volume (between 20 and 150 ml of sample) directional freezing and freeze-drying. Initial experiments have already been performed.

### Author Contributions

Conceived and designed the experiments: DN AA. Performed the experiments: DN AN. Analyzed the data: DN AN AA. Contributed reagents/materials/analysis tools: AN. Wrote the paper: DN AA.

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